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# Reproductive isolation and intraspecific structure in Alpine populations of *Erebia euryale* (Esper, 1805) (Lepidoptera, Nymphalidae, Satyrinae)

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**Abstract.** The subspecies of *Erebia euryale* (Esper, 1805) have been split into three groups based on morphology, differing in male genital characters. Two of them, the *euryale* group and the *adyte* group, are known to be strongly, but not completely, reproductively isolated. There is genetic evidence that their separation preceded the differentiation of subspecies within the *euryale* group. No such data exist on the third group, the recently recognized *kunzi* group. In this study, the degree of reproductive isolation between the *kunzi* group and the other two groups is assessed. In three secondary contact zones, a series of *E. euryale* populations were sampled in a transect perpendicular to the dividing line. Morphological characteristics showed a clinal gradient along each transect. The steepest gradient was found between the *euryale* and *kunzi* groups. Morphologically detectable introgression did not exceed two kilometres. This is comparable to the situation described earlier in contact zones of the *euryale* and *adyte* groups. In the contact area of the *kunzi* and *adyte* groups, the character gradient slope is more gradual and the morphologically detectable introgression zone is at least five times wider. In contrast to this, contact between subspecies belonging to the same group leads to virtually unrestricted morphological intermingling. It is concluded that the *euryale* group is reproductively more strongly isolated from the other two groups than the *kunzi* group is from the *adyte* group, and that subspecies belonging to the same group are interfertile to a high degree. It is argued that loss of genetic compatibility by long term separation is the main cause of the reproductive isolation between groups, and that, consequently, the actual intraspecific structure of *E. euryale* results from at least two, probably three, temporally separated differentiation events.

## Introduction

*Erebia euryale* (Esper, 1805) is a butterfly species with a highly disjunctive distribution and considerable geographic variation. Both its genetic diversity and its distribution pattern have been mainly shaped by climatic fluctuations during the Pleistocene (Schmitt and Haubrich 2008), as was the case in most Palaearctic organisms (Hewitt 1996; Comes and Kadereit 1998; Hewitt 1999, 2000; Kropf et al. 2002; Tribsch and Schönswetter 2003; Schönswetter et al. 2005; Schmitt 2007, 2009). Climate induced range shifts, either latitudinal or altitudinal, repeatedly led to area fragmentation and to retraction or expulsion into glacial refugia. Long-term isolation in these refugia resulted in genetic divergence and, as a consequence, in morphologic differentiation and decreased reproductive compatibility. Each of these three aspects contributed to the actual knowledge of the intraspecific structure of *E. euryale*. Al-

lozyme data suggest a two-level intraspecific structure. Schmitt and Haubrich (2008) determined the genetic distances between eleven *E. euryale* populations. These clustered into four groups. The genetic distances between three of them were roughly equal, while the fourth one was more distant. The authors suggested that this group had split off earlier, and that the observed intraspecific genetic structure has resulted from two subsequent differentiation events. This nested structure was less obvious in a study of mitochondrial DNA (Vila et al. 2011). The morphological structure of *E. euryale* shows two differentiation levels as well. Cupedo (2010) analysed 72 populations, covering all described subspecies, and found them clustering into three groups. These groups differ in male genital anatomy. They are known as the *euryale*, *adyte* and *kunzi* group. The morphological and genetic structuring are concordant: the genetically most distant cluster belongs to the *adyte* group; the three more coherent clusters consistently represent different subspecies of the *euryale* group. The scarce existing data on reproductive isolation fit into this pattern. Representatives of the *euryale* group and the *adyte* group tend to remain separated in secondary contact zones (Rezbanyai-Reser 1991; Sonderegger 2005). Transitional zones are narrow, and hardly contain any hybrids. Some subspecies of the *euryale* group, on the other hand, build transitional zones of up to 40 km in width (Cupedo 2010), mainly consisting of morphological hybrids.

Altogether, genetics, morphology, as well as the degree of reproductive isolation, support the hypothesis of a two-level nested structure of *E. euryale*, at least as far as the *euryale* and *adyte* groups are concerned. Little is known, though, on the *kunzi* group. Morphologically, it has to be ranked in the first level of hierarchical differentiation because it differs considerably from both the *euryale* and *adyte* groups in male genital anatomy. At present, genetic data are lacking, and little is known about contact sites with the other two morphological groups (Cupedo 2010).

The aim of the present study is to assess the degree of reproductive isolation between the *kunzi* group and the other two groups, and to determine whether this is concordant with the morphological traits. For this purpose, three known contact zones, one with the *adyte* group and two with the *euryale* group, were intensively sampled. For each of these contact zones, three questions were addressed: (i) Are hybrid populations present in the contact zone? If so, (ii) what is their composition, and (iii) does their composition show a clinal character gradient across the contact zones? If the latter is found to be the case, the steepness of the cline will provide information regarding the strength of reproductive barriers between the groups. Finally, all available data on reproductive isolation in *E. euryale* are combined in order to establish whether or not they support the hypothesis that two intraspecific differentiation levels exist.

## Material and methods

### *The* kunzi *group*

The *kunzi* group occupies a restricted but well-defined part of the Italian Alps (Fig. 1). Its distribution area comprises (i) the entire pre-Alps between Lake Como and the Valcellina, (ii) the Bergamasque Alps, (iii) the Southern Rhaetian Alps (Ortler, Adamallo-Presanella, Brenta and Nonsberg Alps), and (iv) the Dolomites south of the Latemar-Focobon chain and west of the

Cordevole river. This territory is almost entirely bordered by insurmountable river valleys and mountain chains.

## Contact sites

Secondary contact with populations of other groups requires natural interruptions in this chain of barriers. Four such "exchange windows" exist, three of which were investigated in this study. These are (i) the Falcade region and (ii) the Passo Rolle region, where the *kunzi* group (ssp. *kunzi*) is in contact with the *euryale* group (represented by ssp. *ocellaris*), and (iii) the Trafoi valley and the Sulden valley upstream of their confluence near Gomagoi, where the *kunzi* group (represented by ssp. *pseudoadyte*) meets the *adyte* group. The fourth exchange window, the upper Valtellina (Adda valley), was not sampled.

## Sampling

*E. euryale* has a two year life cycle. In the contact regions, samples were collected in 2009, 2011 and 2013, so the cohorts on the wing were the same in each collecting season. Samples in the Falcade region are labelled F1–F6 (Fig. 2), in the Passo Rolle region R1–R5 (Fig. 3), and in the Trafoi region T1–T5 (Fig. 4). In the Passo Rolle region, special attention was paid

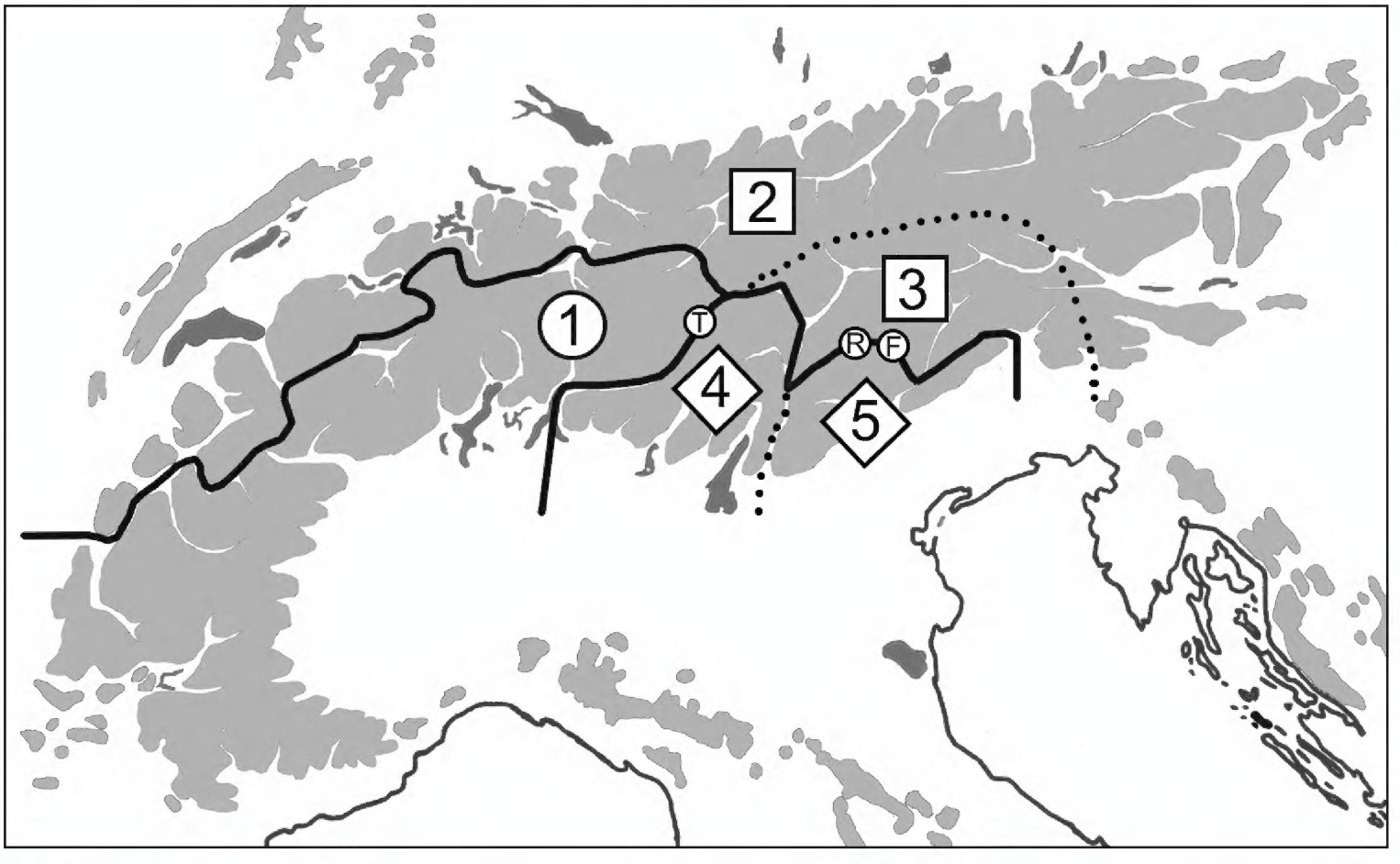



**Figure 1.** The Alps, with geographic boundaries of the taxa of *E. euryale* discussed in this paper. Light grey: mountain areas above 1000 m, dark grey: lakes. Solid lines: group boundaries. Dotted lines: subspecies boundaries. Circle – the *adyte* group, with ssp. *adyte* (1); squares – the *euryale* group with ssp. *isarica* (2) and ssp. *ocellaris* (3); diamonds – the *kunzi* group, with ssp. *pseudoadyte* (4) and ssp. *kunzi* (5). T = Trafoi test region, R = Passo Rolle test region, F = Falcade test region. The intergradation zone *isarica* / *ocellaris* is included in the *ocellaris* area.

to Passo Colbricon. Here, a short transect was sampled and treated as a separate sample set: C1–C3 (Fig. 3).

Samples from the contact regions (called "test samples") were compared to samples from populations outside the contact region ("reference samples"). Each reference sample consists of 150 individuals of one subspecies. These originate from five localities, scattered in the territory, but at a distance of at least 40 km from the exchange regions. In the *adyte* territory, no samples were taken west of Lago Maggiore, since ssp. *adyte* might not be genetically homogeneous in its entire distribution area (Schmitt and Haubrich 2008). For sampling locations and sampling sizes see Figs 2–4 and Table 1.

Female genitalia of different groups are indistinguishable. Female wing pattern enables a certain separation of the ssp. *kunzi* and *ocellaris*, but not of the ssp. *adyte* and *pseudoadyte*. Therefore this study is entirely based on male characters.

## Genital preparation

Male abdominal tips were macerated for 10 min in a 10% KOH solution at 100°C, the genital apparatus was extracted, dehydrated in ethanol (96%) for 10 min, and embedded in euparal.

## Variables

Individual males were characterised on the basis of four variables. Three of these are characteristics of the valve and one is derived from the wing pattern. Valve characteristics were measured on the right valve, as described and figured in Cupedo (2010): 1. shoulder index: the height/width ratio of the dorsal shoulder; 2. first tooth: the relative position of the most proximal tooth; 3. tooth length: the relative length of the longest tooth on the shoulder, as a percentage of the valve length; 4. the presence or absence of discriminating elements in the male wing pattern. A character is regarded discriminating if both its specificity and its positive predictive value are > 0.90, according to the extensive dataset in Cupedo (2010). Discriminating elements are the following (see Table 2): for ssp. *ocellaris* brown ringed ocelli on the hindwing underside; for ssp. *kunzi* white pupils in the ocelli on the forewing upperside, the absence of ocelli on the forewing upperside or on the hindwing upperside, no traces of the brown postdiscal band on the forewing upperside or on the forewing underside. In the case of ssp. *adyte* and ssp. *pseudoadyte*, none of the characters met these criteria. Character 3, on the other hand, does not discriminate between ssp. *kunzi* and ssp. *ocellaris*. Hence, in the contact zone *adyte-pseudoadyte*, characterization of individuals in test populations was based on variables 1, 2 and 3, in the contact zone *kunzi-ocellaris* on variables 1, 2 and 4.

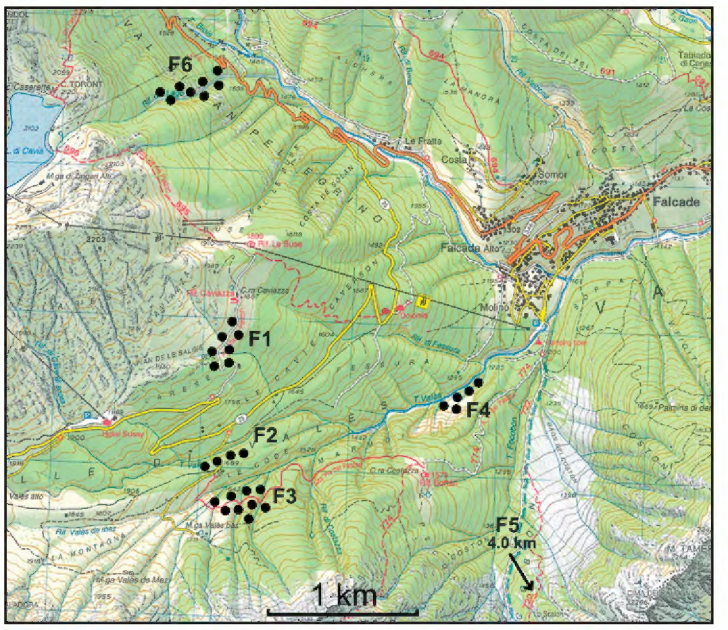
Measurements for shoulder index and first tooth were made using a Mitutoyo 176-902 measuring microscope (magnification 30-fold). Tooth length was measured from calibrated microphotographs on a monitor (final magnification 1000-fold). Variable 4 was assessed with +2 dioptre glasses.

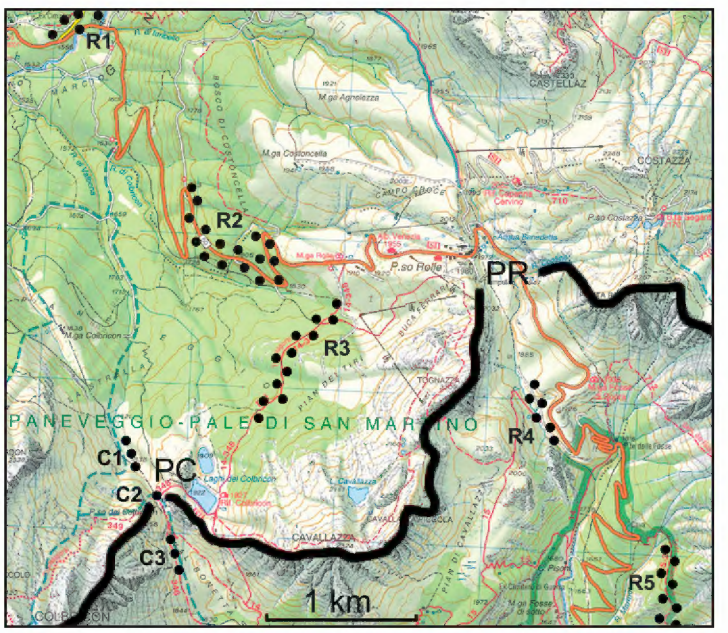
## Scoring system and data analysis

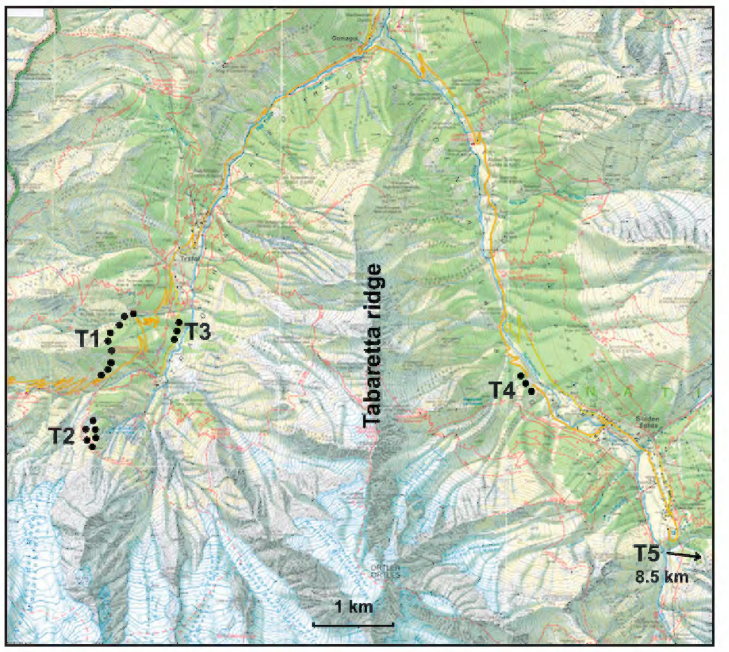
**Characterizing individuals and samples.** A scoring system was developed by which each individual and each sample could be characterised. For each variable, the values of all individuals in both reference groups (e.g. *adyte* and *pseudoadyte*) were combined. The hereby obtained numerical range was split into seven categories, labelled -3 to +3. The centre of the zero category of the scale coincides with the intersection of the frequency distributions of the two reference groups. For each male, the value of each variable was converted into a score, equal to the category it falls into, thus ranging from -3 to +3. Each individual male was characterised by the sum of its scores for the three variables, potentially ranging from -9 (the most *adyte*-like individuals) to +9 (the most *pseudoadyte*-like ones). The scoring procedure was essentially the same in the analysis of the *kunzi* and *ocellaris* samples, except for the fact that variable 3 was replaced by variable 4, which has only three categories: -3 (*ocellaris*), +3 (*kunzi*) or zero (no discriminating wing character present). Each sample was characterised by the frequency distribution of its individual scores.

**Identifying transitional samples.** The frequency distributions of the scores in test samples were compared with those in the reference samples, using the Mann-Whitney U test. A test sample was considered transitional if it differed significantly ($p < 0.05$, two-sided) from both reference samples.

**Test for hybridization.** The question whether a transitional sample contains hybrids requires recognition of hybridization, not necessarily of hybrid individuals. Testing for hybridization was based on the assumption that hybrid butterflies are hardly ever equal to one of their parents: they exhibit either a combination of parental characters, or they have intermediate characters (Mayr 1963). In either




**Figures 2–4.** Topography of the test regions. 2. Falcade test region, 3. Passo Rolle test region and 4. Trafoi test region. Dotted: locations of test populations. Solid line in Figure 3: mountain chain. PR = Passo Rolle; PC = Passo Colbricon. Note the different scale in Figure 4. Reproduced from Tobacco maps 022 (Figures 2 and 3) and 08 (Figure 4).

**Table 1.** Sampling locations, sample codes and sample sizes of the sampled *E. euryale* populations. Code = sample code used in this paper; N = sample size

| Sample location | Code | N | Sample location | Code | N |
|---|---|---|---|---|---|
| ***ssp. adyte*** | **ad** | 150 | ***Test region Falcade*** | | |
| Eggen am Simplon (CH) | | 30 | Falcade-1 | **F1** | 60 |
| Pontresina (CH) | | 26 | Falcade-2 | **F2** | 35 |
| Monte Tamaro (CH) | | 26 | Falcade-3 | **F3** | 60 |
| Langtauferertal (I) | | 30 | Falcade-4 | **F4** | 48 |
| Fusio (CH) | | 38 | Valle di Gares | **F5** | 14 |
| ***ssp. pseudoadyte*** | **ps** | 150 | Valle di Gares | **F5** | 14 |
| Val Malga, Adamello (I) | | 33 | Passo San Pellegrino | **F6** | 15 |
| Monte Baldo (I) | | 30 | ***Test region Passo Rolle*** | | |
| Monte Tremalzo (I) | | 30 | Paneveggio | **R1** | 22 |
| Monte Legnone (I) | | 27 | Passo Rolle road, west | **R2** | 29 |
| Pradalago, Presanella (I) | | 30 | Sentiero laghi di Colbricon | **R3** | 44 |
| ***ssp. kunzi*** | **ku** | 150 | Path Rolle - Colbricon | **R3** | 50 |
| Monte Cavallo (I) | | 47 | Passo Rolle road, east | **R4** | 30 |
| Vette Feltrine (I) | | 30 | San Martino di Castrozza | **R5** | 30 |
| Cimonega (I) | | 30 | Passo Colbricon (north) | **C1** | 37 |
| Col Visentin (I) | | 30 | Passo Colbricon | **C2** | 25 |
| Monte Grappa (I) | | 13 | Passo Colbricon (south) | **C3** | 50 |
| ***ssp. ocellaris*** | **oc** | 150 | ***Test region Trafoi*** | | |
| Geissler Gruppe (I) | | 30 | Trafoi, Madatsch | **T1** | 60 |
| Sesto (I) | | 30 | Trafoi, left bank | **T2** | 60 |
| Plöckenpass (I) | | 30 | Trafoi, south of camping | **T3** | 39 |
| Passo Fedaia (I) | | 30 | Sulden, south of Karnerbrücke | **T4** | 60 |
| Lienzer Dolomites (A) | | 30 | Martelltal, Lify alm | **T5** | 60 |

case, the scores tend to drift to zero, i.e. towards lower absolute values. To test for such shift, category labels were made absolute, and the values of identical categories were added (-9 and + 9 became 9, and the numbers in the categories -9 and +9 were added in category 9, etc.). The cumulative graph of the values thus obtained (hereafter called the "absolute graph") was compared to the graph of an imaginary cohabitational population without hybridization, created by combining both reference samples. If a test sample contains hybrids, its graph will show a left shift when compared to the reference graph. The magnitude of such a shift is an empirical estimation of the degree of hybridization in the transitional population. The procedure is illustrated in Fig. 5.

**Test for clinal variation.** In each test sample, the proportions of individuals with a negative score and with a positive score were calculated. This provides a good approximation of the proportions of parental characters in the population, without the need of individual identification, and regardless of the hybridization rate in the population. These proportions were used to detect geographic clines in the contact zones. Because of the small overlap of the distributions of scores in the *adyte* and *pseudoadyte* reference samples, 3.3% of the positive scores are incorrectly classified as *pseudoadyte*, and 5.3% of the negative scores are incorrectly classified as *adyte*. The more one of both types is predominant, the more this will affect the

**Table 2.** Specificity (sp) and positive predictive value (ppv) of characters discriminating between the subspecies *ocellaris* and *kunzi* of *E. euryale*. * –Data underlying Table 4 in Cupedo (2010). Abbreviations: Hw = hindwing; Fw = forewing; Up = upperside; Un = underside; Oc = apical ocelli; B = brown postdiscal band.

| ssp | character | value | | # true* | # false* | sp<br>ppv | 95% confidence interval |
|---|---|---|---|---|---|---|---|
| *ocellaris* | HwUnOc | with brown ring | pos | 314 | 7 | 0.9857 | 0.9708-0.9940 |
| | | | neg | 54 | 484 | 0.9782 | 0.9556-0.9911 |
| *kunzi* | FwUpOc | with white pupil | pos | 183 | 3 | 0.9918 | 0.9763-0.9982 |
| | | | neg | 308 | 365 | 0.9839 | 0.9535-0.9965 |
| *kunzi* | FwUpOc | absent | pos | 78 | 7 | 0.9810 | 0.9612-0.9923 |
| | | | neg | 413 | 361 | 0.9176 | 0.8376-0.9661 |
| *kunzi* | HwUpOc | absent | pos | 323 | 30 | 0.9158 | 0.8857-0.9443 |
| | | | neg | 167 | 338 | 0.9150 | 0.8809-0.9419 |
| *kunzi* | FwUpB | absent | pos | 157 | 4 | 0.9891 | 0.9724-0.9970 |
| | | | neg | 334 | 364 | 0.9752 | 0.9375-0.9930 |
| *kunzi* | FwUnB | absent | pos | 115 | 0 | 1.0000 | 0.9899-1.0000 |
| | | | neg | 376 | 368 | 1.0000 | 0.9681-1.0000 |

*adyte* / *pseudoadyte* ratio. As a result, any clinal character gradient in the *adyte* / *pseudoadyte* contact zone will be slightly underestimated.

### Statistics

Statistical tests were performed with the SPSS 12.0 package. Specificity and positive predictive value of discriminating characters were calculated with MedCalc online statistical calculators for Windows, version 12.7.8.

### Material deposition

Samples and genital preparations are deposited in the collection of the author.

## Results

### Identifying transitional samples

The distributions of individual scores per sample are shown in Table 3. The results of a statistic comparison of these distributions (Mann Whitney test), with each other and with the reference samples, for each of the contact zones, are presented in Fig. 6. In each contact zone there were (i) test samples that did not differ significantly either from each other or from one of the reference samples, but did differ from the second reference sample (grey in Fig. 6), and (ii) test samples that differed significantly from both reference samples and from the test samples mentioned under (i). The latter were considered transitional. These were: F2, F3 and F4 in the Falcade region; R3, C1 and C2 in the Passo Rolle region and T3 and T4 in the Trafoi region.

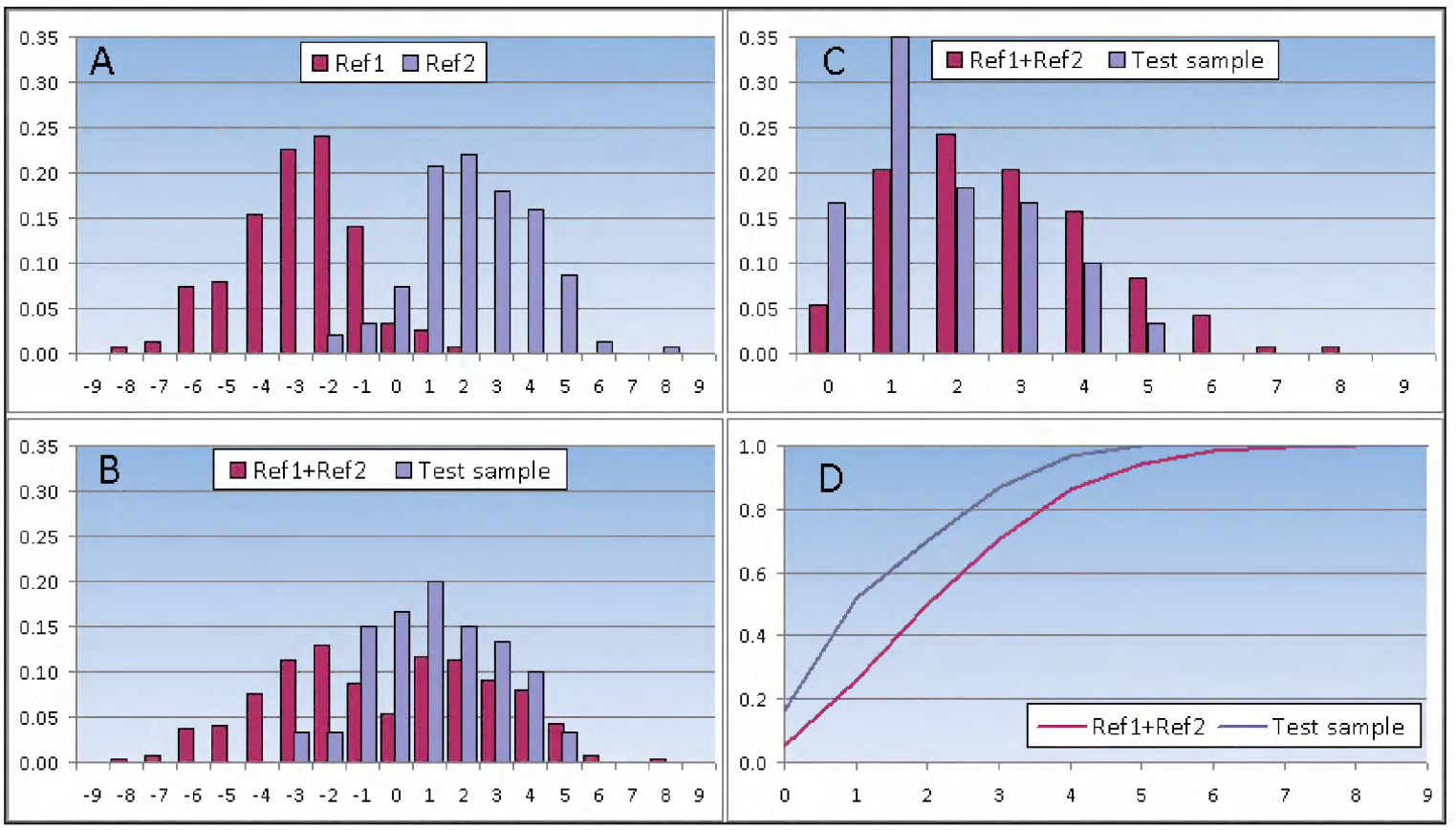



**Figure 5.** Illustration of the procedure for detection of hybrids. A: frequency distributions of scores in reference samples; B: frequency distribution of scores in the combined reference samples, and in a test sample; C: as B, X-axis categories are made absolute. D: as C, cumulative. X-axis: score (A and B) or absolute score (C and D); Y-axis: proportion of the sample.

**Test for hybridization in transitional samples**

In Fig. 7, the absolute graphs of all samples are compared to the pooled reference samples. A left shift is obvious in all transitional samples and, to a much lower degree, in some samples that are not classified as transitional. This implies that intergroup mating produced adult hybrids in each of the studied contact zones.

***The composition of the transitional samples***

The score distributions of the transitional samples in the hybrid zones *kunzi* / *ocellaris* are different from those in the hybrid zone *adyte* / *pseudoadyte* (Fig. 8). In the former, the ranges almost cover the combined ranges of both reference samples. F2, R3 and C2 show an explicit bimodal distribution; in F3, the distribution is strongly biased towards the *kunz*i phenotype. In the contact area *adyte* / *pseudoadyte*, on the other hand, the range of the individual scores covers no more (T3) or hardly more (T4) than the range of one of the reference samples. Typical *pseudoadyte* individuals are lacking in T3, and typical *adyte* are absent from T4. The distributions are unimodal, but both are skewed towards the hybrid end of the scale.

***Test for clinal variation in the transition zones***

The fractions of negative and positive scores (Table 4) exhibited a clinal gradient in each of the contact zones. In the Falcade region, this is along the (F6-F1)-F2-F3-F5 line; in the Passo Rolle region along the R1-R2-R3-(R4-R5) line and along C1-C2-C3, and in the Trafoi test region

**Table 3.** Distributions of individual scores per sample, in reference samples and test samples of *E. euryale*.

| | | **-9** | **-8** | **-7** | **-6** | **-5** | **-4** | **-3** | **-2** | **-1** | **0** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **N** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Reference | **ad** | | 1 | 2 | 11 | 12 | 23 | 34 | 36 | 21 | 5 | 4 | 1 | | | | | | | | 150 |
| samples | **ps** | | | | | | | | 3 | 5 | 11 | 31 | 33 | 27 | 24 | 13 | 2 | | 1 | | 150 |
| | **oc** | | 7 | 15 | 46 | 41 | 25 | 9 | 5 | 2 | | | | | | | | | | | 150 |
| | **ku** | | | | | | | | | | | 4 | 1 | 12 | 23 | 33 | 47 | 27 | 2 | 1 | 150 |
| Falcade | **F1** | | 3 | 12 | 17 | 9 | 7 | 6 | 3 | 1 | 1 | | 1 | | | | | | | | 60 |
| | **F2** | | 1 | 6 | 1 | 2 | 2 | 3 | 1 | | | 1 | 1 | 3 | 4 | 4 | 2 | 3 | 1 | | 35 |
| | **F3** | | | | 1 | | | 2 | | 2 | 2 | 3 | 4 | 10 | 10 | 17 | 4 | 4 | 1 | | 60 |
| | **F4** | | | 1 | 1 | 2 | 1 | 2 | 5 | 1 | 5 | 4 | 3 | 6 | 3 | 6 | 5 | 2 | 1 | | 48 |
| | **F5** | | | | | | | | | | | | 2 | 1 | 2 | 3 | 3 | 1 | 2 | | 14 |
| | **F6** | | 1 | 4 | 4 | 4 | 1 | | | | 1 | | | | | | | | | | 15 |
| Passo | **R1** | | | 2 | 6 | 8 | 2 | 3 | | 1 | | | | | | | | | | | 22 |
| Rolle | **R2** | 1 | | 2 | 8 | 8 | 2 | 3 | 2 | 2 | | 1 | | | | | | | | | 29 |
| | **R3** | | 1 | 4 | 8 | 5 | 1 | 6 | 10 | 1 | 2 | 2 | 1 | | | 5 | 3 | 1 | | | 50 |
| | **R4** | | | | | | | | | | | | 1 | 4 | 6 | 9 | 5 | 3 | 2 | | 30 |
| | **R5** | | | | | | | | | | | | 3 | 2 | 6 | 9 | 5 | 5 | | | 30 |
| | **C1** | | 1 | 5 | 3 | 5 | 2 | 5 | | 1 | 4 | 2 | 1 | 1 | 2 | 3 | 1 | 1 | | | 37 |
| | **C2** | | 1 | | 2 | 3 | 1 | | 1 | 1 | | 1 | | 4 | 2 | 3 | 4 | 2 | | | 25 |
| | **C3** | | | | | | | | | | | | 1 | 3 | 10 | 16 | 12 | 6 | | 2 | 50 |
| Trafoi | **T1** | | | 1 | 3 | 6 | 6 | 12 | 15 | 7 | 6 | 4 | | | | | | | | | 60 |
| | **T2** | | | | 6 | 4 | 11 | 11 | 13 | 9 | 3 | 1 | 2 | | | | | | | | 60 |
| | **T3** | | | | | 4 | 4 | 8 | 2 | 8 | 4 | 4 | 5 | | | | | | | | 39 |
| | **T4** | | | | | | | 2 | 2 | 9 | 10 | 12 | 9 | 8 | 6 | 2 | | | | | 60 |
| | **T5** | | | | | | | | 1 | 3 | 8 | 9 | 14 | 8 | 8 | 6 | 2 | 1 | | | 60 |

along (T1-T2)-T3-T4-T5. Samples in brackets do not differ significantly from each other ($p < 0.05$, two-sided).

## Discussion

### The composition of the hybrid samples

Transitional samples were present in each of the three contact zones, and in each of them hybridization took place, so the contact zones discussed in this paper explicitly are hybrid zones. There is, however, a noticeable variation in the score distributions of the test samples, both among and within contact areas. Field observations yield enough additional information to explain these differences.

*1. The Falcade test region*. In the Falcade contact zone, the northern slopes of the Valle di Vales are inhabited by ssp. *ocellaris*, whereas ssp. *kunzi* occupies the southern slopes. Samples F1 and F3 were taken from high-density populations, living in clearings in a mixed fir-larix forest, with F1 at the upper tree limit on the northern slope (1870 m), and F3 in the lower part of the opposite slope (1670 m). In between, individuals of *E. euryale* were scarce, flying along roadsides and on hay meadows. A local concentration was only found at location

| A | oc | F6 | F1 | F2 | F3 | F4 | F5 | ku |
|---|---|---|---|---|---|---|---|---|
| oc | | ns | ns | *** | *** | *** | *** | *** |
| F6 | | | ns | ** | *** | *** | *** | *** |
| F1 | | | | ** | *** | *** | *** | *** |
| F2 | | | | | ** | ns | ** | *** |
| F3 | | | | | | ** | * | *** |
| F4 | | | | | | | ** | *** |
| F5 | | | | | | | | ns |
| ku | | | | | | | | |

| B | ad | T1 | T2 | T3 | T4 | T5 | ps |
|---|---|---|---|---|---|---|---|
| ad | | ns | ns | ** | *** | *** | *** |
| T1 | | | ns | * | *** | *** | *** |
| T2 | | | | ** | *** | *** | *** |
| T3 | | | | | *** | *** | *** |
| T4 | | | | | | ** | *** |
| T5 | | | | | | | ns |
| ps | | | | | | | |

| C | oc | R1 | R2 | R3 | R4 | R5 | ku |
|---|---|---|---|---|---|---|---|
| oc | | ns | ns | *** | *** | *** | *** |
| R1 | | | ns | ** | *** | *** | *** |
| R2 | | | | ** | *** | *** | *** |
| R3 | | | | | *** | *** | *** |
| R4 | | | | | | ns | ns |
| R5 | | | | | | | ns |
| ku | | | | | | | |

| D | oc | C1 | C2 | C3 | ku |
|---|---|---|---|---|---|
| oc | | *** | *** | *** | *** |
| C1 | | | * | *** | *** |
| C2 | | | | ** | *** |
| C3 | | | | | ns |
| ku | | | | | |

**Figure 6.** Mann-Whitney's significance levels for pair wise comparison of the frequency distributions of the scores of all samples. A: Falcade test region; B: Trafoi test region; C: Passo Rolle test region; D: Passo Colbricon. For sample codes see Table 1. Legend for *p* values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant.

F2. The contact zone, which was sampled in 2009, was revisited in 2013. The situation at F1 and F3 was unaltered. In between, though, more individuals were present than in 2009, but no concentration was observed at site F2. This gives the impression of two stable, permanent populations (F1 and F3), from where individuals swarm out, annually, into the less suitable zone in between. This would explain the temporary character of F2, as well as its bimodal distribution. The score distribution of F1 does not differ significantly from the *ocellaris* reference sample, and F3 shows a *kunzi*-like distribution, which tails out on the left side, evidencing hybridization with *ocellaris*. Apparently, down slope roaming (F1 → F3) exceeds uphill movement in this locality.

One kilometre downstream, the valley floor (at 1250 m) was inhabited by the hybrid population F4. Due to the inaccessibility of the slopes here, no migration was actually observed, but the composition of the population strongly supports a regular influx from both sides. Given the

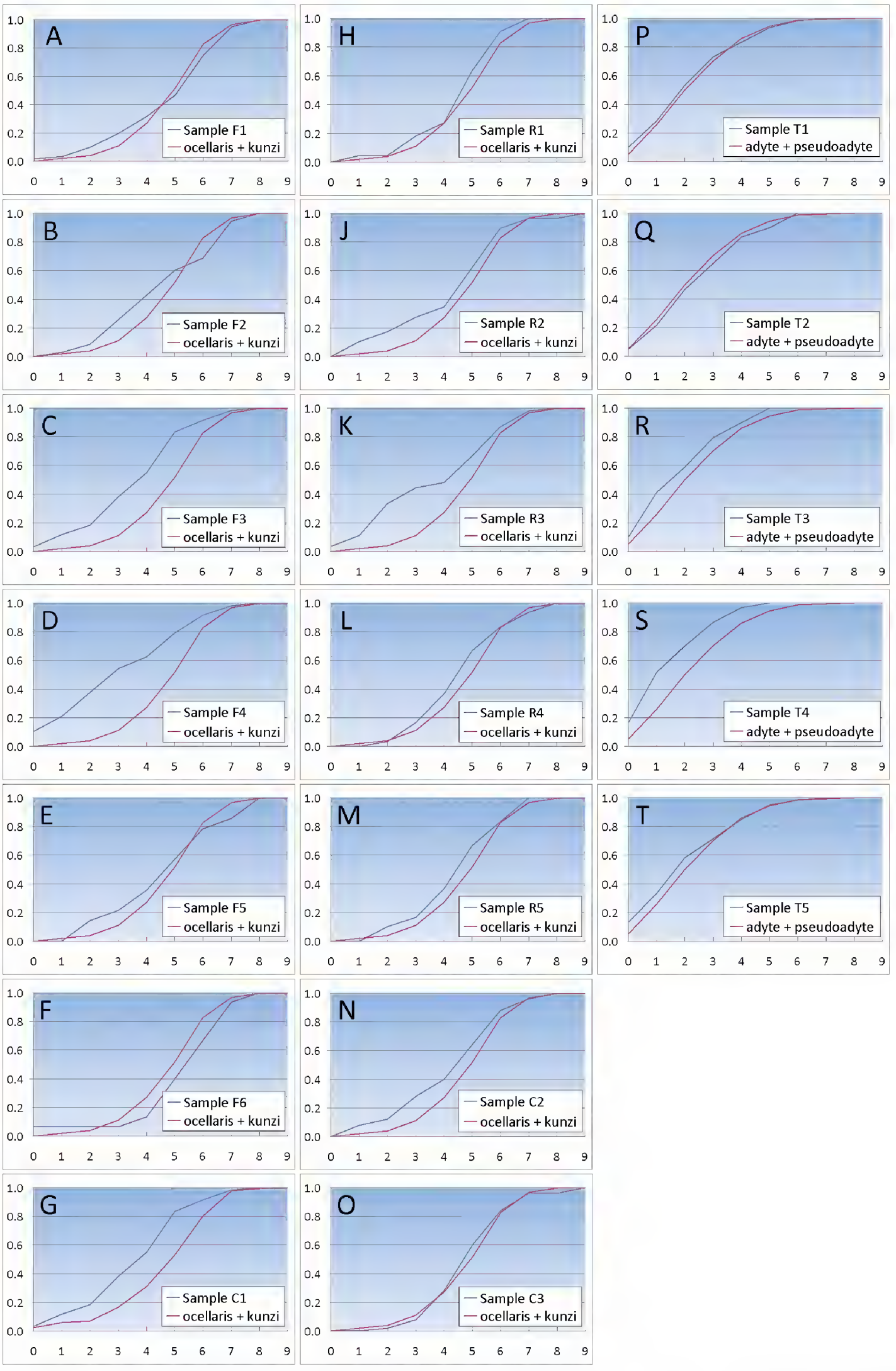



**Figure 7.** Test for the presence of hybrids in test samples of *E. euryale* in contact zones between groups. X-axis: absolute score; Y-axis: proportion of the sample. For sample codes see Table 1.

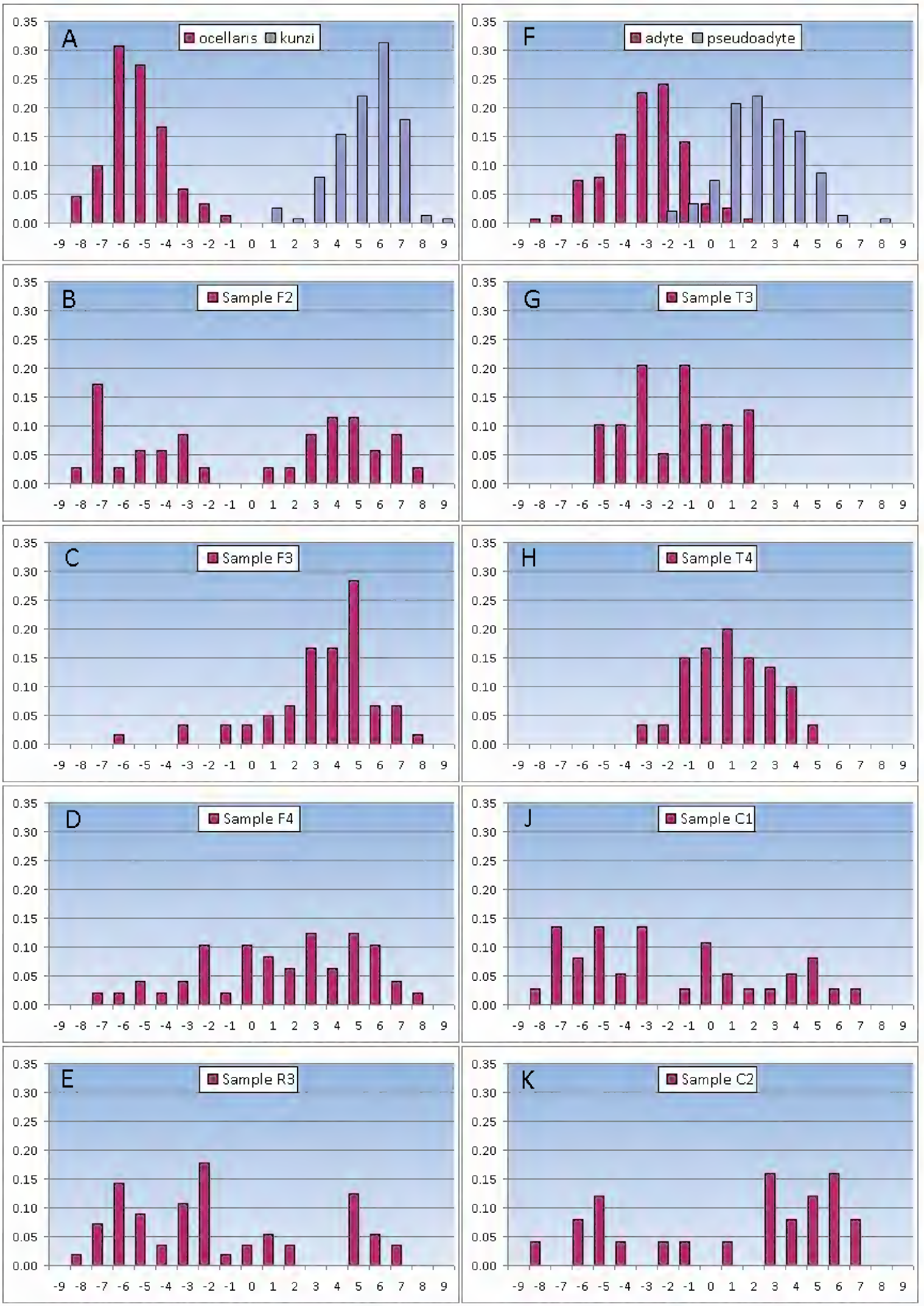



**Figure 8.** Frequency distribution of individual scores in reference samples (A, F) and in transitional test samples of *E. euryale*. X-axis: score; Y-axis: proportion of the sample. For sample codes see Table 1.

**Table 4.** Proportions of individuals with a negative and with a positive score, in each of the *E. euryale* samples.

| | Reference samples | | | | | Passo Rolle | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Scores** | **ad** | **ps** | **oc** | **ku** | | **R1** | **R2** | **R3** | **R4** | **R5** | **C1** | **C2** | **C3** |
| **<0** | 0.93 | 0.05 | 1.00 | 0.00 | | 1.00 | 0.97 | 0.72 | 0.00 | 0.00 | 0.59 | 0.36 | 0.00 |
| **>0** | 0.03 | 0.87 | 0.00 | 1.00 | | 0.00 | 0.03 | 0.24 | 1.00 | 1.00 | 0.30 | 0.64 | 1.00 |
| | **Falcade** | | | | | | | **Trafoi** | | | | | |
| **Scores** | **F1** | **F2** | **F3** | **F4** | **F5** | **F6** | | **T1** | **T2** | **T3** | **T4** | **T5** | |
| **<0** | 0.97 | 0.46 | 0.08 | 0.27 | 0.00 | 0.93 | | 0.83 | 0.90 | 0.67 | 0.22 | 0.07 | |
| **>0** | 0.02 | 0.54 | 0.88 | 0.63 | 1.00 | 0.00 | | 0.07 | 0.05 | 0.23 | 0.62 | 0.80 | |

high population density in a favourable habitat, this population has to be considered a permanent and breeding population. This might explain why the hybrid ratio is the highest among the analysed hybrid populations.

*2. The Passo Rolle test region.* In the Passo Rolle region, it is the Latemar chain and its continuation, the Focobon chain, that separate *kunzi* from *ocellaris*. Two depressions in this chain, the Passo Rolle and the Passo Colbricon, are potential exchange windows. The largest one, Passo Rolle, is an ecologically devastated area, which offers no suitable habitat to *E. euryale*. A single specimen was observed. Exchange of individuals takes place over the much narrower Passo Colbricon, 2.5 km southwest of Passo Rolle. Here, *E. euryale* was present in relatively high density, on the pass and on both sides. In this continuous population, connecting the *ocellaris* area with the *kunzi* area, an extra set of three samples was taken from nearby sites. C2 was collected on the pass (within 20 metres around the pass mark, Fig. 9), C1 200 to 350 m to the North of the pass and C3 between 200 and 400 m from the pass at its southern slope. This southern sample did not differ significantly from the *kunzi* reference sample, nor was there any detectable hybridization (Table 3, Fig. 7). On the pass, the *ocellaris* portion was 36%, in the more northern C1 sample it was already 59%. This justifies three conclusions: the Colbricon pass, despite its small size, is an important exchange corridor between *kunzi* and *ocellaris*; dispersal is predominantly northward, as C1 and C2 contained a substantial portion of *kunzi*, but *ocellaris* was absent from C3; and maintenance of this sharp separation within a continuous population requires a strong reproductive isolation mechanism.

*3. The Trafoi test region.* In the Trafoi contact zone, *adyte* is widespread west of the Trafoi valley, and *pseudoadyte* occurs east of the Sulden valley. The Tabaretta chain of the Ortler Massif is inserted in between (Fig. 4). The hybrid populations T3 and T4 were found at its foot. The distance T3–T4 is 4.5 km in a straight line (across the Tabaretta ridge, 2800 m), and 8 km when measured along the 2000 m contour line (the most probable migration path). Both distances by far exceed the mean individual range of flight. Gene exchange therefore might take several generations, so parental individuals from T3 will normally not reach T4 and *vice versa*. However, the influx of hybrid phenotypes biases the score distributions of both populations towards the hybrid end of the scale (compare Figs 8G and 8H to Fig. 8F).

**Figure 9.** Passo Colbricon (1908 m), an important exchange corridor of ssp. *kunzi* and ssp. *ocellaris*. View to the south (31.vii.2013).

## Clinal gradients in hybrid zones

In each of the contact zones, a clinal gradient of characters is obvious (Table 4). Because morphologic and genetic clines have been shown to be coincident and concordant (Barton & Hewitt 1985; Collins et al. 1993; Dasmahapatra et al. 2002), the morphologic gradient can be considered to reflect genetic intergradation. Consequently, the rate of introgression can be estimated from the steepness of the morphological gradient.

*The kunzi group and the euryale group.* In the Falcade contact area, the fraction of *ocellaris* characters drops from 0.97 to 0.08 between F1 and F3, and the fraction of *kunzi* characters from 0.88 to 0.02 in the opposite direction (Table 4). That is a mean decline of 87.5% across one kilometre. In the Passo Rolle region, it is 97% across two kilometres (between R2 and R4), and on the Colbricon pass it is 65% over 400 m. These declines are extremely steep, especially when considering that the distances between adjacent samples in all cases are within the normal individual range of flight. In both contact areas, explicit hybrid samples exist (F2, R3, C1, C2), but the more peripheral samples are hardly affected. Morphologically detectable introgression fades out within two kilometres.

*The kunzi group and the adyte group.* In the Trafoi region, the mean decline is 42% over at least 4.5 km, more probably 8 km. Due to the different spacing of sampling, these data cannot directly be compared to those from the *kunzi* / *ocellaris* contact regions. Nonetheless, if either

in the Falcade or in the Passo Rolle region two populations had been analysed 4.5 km apart, hardly any morphological evidence of introgression was to be expected. In the Trafoi region, though, the decline over this distance is only 42%. This at least justifies the conclusion that introgression between the *kunzi* group and the *adyte* group is less inhibited by reproductive barriers than between the *kunzi* group and the *euryale* group.

*The euryale group and the adyte group.* Rezbanyai-Reser (1991) described three contact sites of the *euryale* group (ssp. *isarica*) and the *adyte* group (ssp. *adyte*) in Switzerland: Hasliberg (BE), Gitschen (UR) and Rophaien (UR). His observations are based on wing characters only, and quantitative data are lacking. In each of these localities, he found a transition zone less than 100 m in width. Intermediate individuals flew together with both parental forms, in the absence of any natural barrier. This is fully comparable to the situation in the Falcade and Passo Rolle regions. In one of his localities, Rezbanyai-Reser (1991) made the important observation that the situation remained unaltered over nine years. In other words, no progress of introgression was observed. Hybrid individuals seemed to be generated *de novo* in each generation. Sonderegger (2005) described two comparable situations in Switzerland, based on both wing pattern and valve characters. At Klosters (GR), a cohabitation zone of about 1 km was found. Only at one specific site in this range, intermediate individuals were found, flying together with both parental forms. At Monstein (GR), a cohabitation site without intermediates was observed. An entirely different situation was recorded in two other sites (Brusons, VS and Grindelwald, BE). Here, the ssp. *isarica* and ssp. *adyte* areas are spatially separated, and Sonderegger found an intermediate population in between. According to Mayr (1942), this is a recurrent phenomenon when hybrid populations are deprived of parental influx over a long period of time. Mayr (1942) states that these populations may achieve phenotypic stability by continuous selection against the most unbalanced hybrid genomes.

*Subspecies within groups.* Hybrid zones of two subspecies of *E. euryale* belonging to the same group are rare, since most of them have allopatric distributions. It is only in the Pyrenees and in the Alps that two subspecies of the same group (the *euryale* group) are in secondary contact. In both cases, one of the two subspecies is strongly melanistic, which enables easy identification of hybrid individuals by wing pattern. The hybrid zone in the Pyrenees is insufficiently documented, but it covers a considerable part of the Pyrenees (pers. obs.). The hybrid zone in the Alps (ssp. *isarica* and ssp. *ocellaris*) has been mapped (Cupedo 2010). All populations in the intergradation zone mainly consist of hybrid individuals; parental types are rare or absent. The hybrid zone attains its maximum width of about 40 km in the eastern Alps. More important than its actual width is the fact that, in contrast to the hybrid zones between groups, introgression between these intra-group subspecies proceeded until further dispersal was inhibited by natural barriers (mountain chains in the north and river valleys in the south). Obviously there is a discrepancy between the narrow but stable hybrid zones between groups, and the freely expanding hybrid zone between within-group subspecies.

## The cause of the difference between hybrid zones

More or less stable transition zones between genetically distinct populations have been described in a great variety of organisms (Mayr 1963; Barton & Hewitt 1985; Jiggins & Mallet 2000; Arnold 2006; Schmitt & Müller 2007; Schmitt et al. 2007). They range from some

hundreds of metres to some hundreds of kilometres. Barton and Hewitt (1985) showed that the width of hybrid zones strongly depends on the balance of two antagonistic factors: dispersal and selection. Immigration into the region of overlap tends to widen the transition zone but is continuously counteracted by selection. This selection may be either ecological, i.e. by differences in the environment or by different adaptations to the environment, or genetic, by selection against recombinant genotypes (Barton & Hewitt 1985; Arnold 2006). In the contact sites studied in this paper, ecological factors can be ruled out as selecting factors. The habitats at both sides of the intergradation zones are largely identical, and there are no indications of different ecological preferences among Alpine subspecies of *E. euryale*. Consequently, genetic selection, i.e. a decreased genomic compatibility of the populations in contact, is the most likely factor determining the width of the hybrid zones in *E. euryale*. This selection may be pre-zygotic, by assortative mating, or post-zygotic, by reduced hybrid fertility or viability. In the case of *E. euryale*, the occurrence of hybrids is a sign of random or at least incompletely assortative mating, and the low introgression rate is an indication of reduced hybrid fertility or viability.

Considering the width of the introgression zone, we should keep in mind that morphological markers are far less sensitive than genetic ones. Barton and Hewitt (1985) showed that gradients in secondary contact zones are s-shaped, and that introgression tails out in both directions. Morphologically, these tails will remain hidden because of a lack of resolution. In general, the width of the intergradation zone will be underestimated when based on morphology alone. Geiger and Rezbanyai (1982) have already demonstrated this phenomenon. They found a significantly lower genetic distance (Nei 1972) between *adyte* and the *isarica* population at Hasliberg (one of the Swiss contact sites) than between the same *adyte* population and a more remote, but morphologically identical *isarica* population (D=0.036 and 0.073 respectively!). This demonstrates gene flow by introgression, which could not be detected morphologically.

### *Differentiation levels*

This study of hybrid zones reveals that strong reproductive barriers exist between the *euryale* group and both the *adyte* group and the *kunzi* group. Our results suggest a less strong reproductive isolation of the *adyte* group and the *kunzi* group, but the different spacing of the test samples and the different characters used to discriminate between the groups impede an unambiguous numeric comparison of the results. Reproductive barriers between the subspecies *isarica* and *ocellaris*, both belonging to the *euryale* group, are so weak that they suggest random mating and a high hybrid viability. Consequently, at least two, maybe three, hierarchical levels of reproductive isolation exist between *E. euryale* populations. Since the degree of reproductive isolation is positively correlated with genetic distance, i.e. the duration of the interruption of gene flow (Coyne & Orr 1997; Jiggins & Mallet 2000), the results support the idea of at least two, maybe three, temporally separated differentiation events. In the latter case, disjunction of the *euryale* group and the *adyte-kunzi* precursor would have preceded the disjunction of the *adyte* group and the *kunzi* group. This scenario would be in agreement with the morphological structure, as the *adyte* group and the *kunzi* group are more similar to each other, both in male genital features and in wing pattern, than either is to the *euryale* group.

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